HPTPbeta as a target in treatment of angiogenesis mediated disorders

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CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the priority under Title 35 U.S. Code 119(e) from Provisional Application Serial No. 60/413,547 filed September 25, 2002, which is herein incorporated by reference in its entirety.

FIELD OF INVENTION

This invention is directed to the use of HPTPbeta to screen agents useful in the treatment angiogenesis mediated disorders.

BACKGROUND OF THE INVENTION

Angiogenesis, the sprouting of new blood vessels from the pre-existing vasculature, plays a crucial role in a wide range of physiological and pathological processes (Nguyen, L.L. et al, Int. Rev. Cytol. 204, 1-48, (2001). It is a complex process that is mediated by communication between the endothelial cells that line blood vessels and their surrounding environment. In the early stages of angiogenesis, tissue or tumor cells produce and secrete pro-angiogenic growth factors in response to environmental stimuli. These factors diffuse to nearby endothelial cells and stimulate receptors that lead to the production and secretion of proteases that degrade the surrounding extracellular matrix (Stetler-Stevenson,W.G., Surg. Oncol. Clin. N. Am. 10, 383-392, (2001). These activated endothelial cells begin to migrate and proliferate into the surrounding tissue toward the source of these factors. Endothelial cells then stop proliferating and differentiate into tubular structures, which is the first step in the formation of stable, mature blood vessels. Subsequently, periendothelial cells, such as pericytes and smooth muscle cells, are recruited to the newly formed vessel in a further step toward vessel maturation.

There are many disease states driven by unregulated angiogenesis that can either cause a particular disease directly or exacerbate an existing pathological condition. For example, ocular neovascularization has been implicated as the most common cause of blindness and underlies the pathology of approximately 20 eye diseases. In certain previously existing conditions such as

arthritis, newly formed capillary blood vessels invade the joints and destroy cartilage. In diabetes, new capillaries formed in the retina invade the vitreous humor, causing bleeding and blindness.

On the other hand, tissue growth and repair are biologic systems wherein cellular proliferation and angiogenesis occur. Thus an important aspect of wound repair is the revascularization of damaged tissue by angiogenesis. Atherosclerotic lesions in large vessels can cause tissue ischemia that could be ameliorated by modulating blood vessel growth to the affected tissue. For example, atherosclerotic lesions in the coronary arteries cause angina and myocardial infarction that could be prevented if one could restore blood flow by stimulating the growth of collateral arteries. Similarly, atherosclerotic lesions in the large arteries that supply the legs cause ischemia in the skeletal muscle that limits mobility that could also be prevented by improving blood flow with angiogenic therapy.

In view of the foregoing, there is a need to identify biochemical targets in the treatment of angiogenesis mediated disorders. However, angiogenesis involves the action of multiple growth factors and their cognate receptor tyrosine kinases (RTKs), Yancopoulos et al., Nature, 407,242-248, 2000). Vascular endothelial growth factor (VEGF), for example, is critical for the differentiation of endothelial cells into nascent blood vessels in the embryonic vasculature. Further, VEGF enhances blood vessel development in the adult vasculature. Administration of exogenous VEGF enhances the development of the collateral vasculature and improves blood flow to ischemic tissues.

To date, three VEGF RTKs have been identified, VEGFR1 (FLT-1), VEGFR2 (KDR), and VEGFR3 (FLT-4). Although these receptors are highly conserved, based on biochemical characterization and biological activity, each has specific and non-overlapping functions. Of the three receptors, VEGFR2 plays the predominant role in mediating VEGF actions in the developing vasculature and during angiogenesis in adults. However, both VEGFR1 and VEGFR3 are required for normal development of the embryonic vasculature and may also be important for angiogenesis in adult tissues. Upon VEGF binding and dimerization, a conformational change in the VEGFR2 kinase domain enhances its kinase activity resulting in "autophosphorylation" of the other member of the pair on specific tyrosine residues. These autophosphorylation events serve to further enhance the kinase activity and provide anchor points for the association of intracellular signaling molecules.

However, activation of a single angiogenic pathway may not be sufficient to produce persistent and functional vessels that provide adequate perfusion to ischemic tissue. These findings, together with fact that multiple RTKs are involved in the assembly of embryonic vasculature,

indicate that biochemical targets that modulate multiple angiogenic pathways will have advantages over administration of a single growth factor.

Protein tyrosine phosphatases (PTPs) comprise a large family of closely related enzymes that dephosphorylate proteins that contain phosphotyrosine residues. Recent evidence suggests that one function of PTPs is to limit the phosphorylation and activation of RTKs. For example, HCPTPA, a low molecular weight protein tyrosine phosphatase, was shown to associate with VEGFR2 and negatively regulate its activation in cultured endothelial cells and its biological activity in angiogenesis assays, (Huang et al., Journal of Biological Chemistry, 274, 38183-38185, 1999). Whether or not other PTPs might regulate VEGFR2 activation is not known.

In addition to VEGFR2, signaling input from another RTK Tie-2, the receptor for the angiopoietins (Ang1 and Ang2), is also essential. Deletion of either Ang1 or Tie-2 gene in mice results in embryonic lethality secondary to abnormalities in the developing vasculature (Yancopoulos et al., Nature, 407, 242-248, 2000). In addition, overexpression of Ang1 in the skin increases skin vascularity and administration of exogenous Ang1 increases blood flow to ischemic skeletal muscle (Suri et al., Science, 282, 468-471, 1998). Moreover, inhibiting the activation of Tie-2 inhibits angiogenesis and limits tumor progression in animal models of cancer, (Lin et al., JCI, 100, 2072-2078, 1997). In addition to its angiogenic activities, activation of Tie-2 by exogenous administration of Ang1 blocks VEGF mediated vascular leak and pro-inflammatory effects, but enhances its angiogenic effects (Thurston et al., Nature Medicine, 6, 460-463, 2000). Therefore, biological targets that modulate both VEGFR2 and Tie-2 signaling may yield superior proangiogenic or antiangiogenic therapies.

SUMMARY OF THE INVENTION

The present invention is based, in part, on the discovery that modulation of HPTPbeta activity modulates the activation and the biological activity of both VEGFR2 and Tie-2. As such, the present invention identifies and provides HPTPbeta as a target to identify agents useful in the treatment of angiogenesis mediated disorders.

In one aspect, the invention provides for a method of identifying an agent useful for modulating angiogenesis comprising the steps of: (a) exposing HPTPbeta to an agent; (b) measuring activity of HPTPbeta, wherein modulation of HPTPbeta activity indicates the agent is useful for modulating angiogenesis.

In another aspect, the invention provides for a method of identifying an agent useful for modulating angiogenesis comprising the steps of: (a) exposing HPTPbeta to an agent to form HPTPbeta/agent complex; (b) exposing the HPTPbeta/agent complex to VEGFR2; and (c) measuring the activity of VEGFR2; wherein a modulation in VEGFR2 activity indicates the agent is useful for modulating angiogenesis.

In another aspect, the invention provides for a method of identifying an agent useful for modulating angiogenesis comprising the steps of: (a) exposing HPTPbeta to an agent; (b) exposing HPTPbeta/agent complex to VEGFR2 and Tie-2; and (c) measuring the activity of VEGFR2 and/or Tie-2; wherein a modulation in VEGFR2 and/or Tie-2 activity indicates the agent is useful for modulating angiogenesis.

The above-described embodiments may be performed in vitro, either in cell free or cell-based assays; or in vivo, or ex vivo using tissue implants.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Schematic representation of the 5' end of the HPTPbeta gene showing that the missing exon codes for FNIII repeat 5 compared to Genbank entry X54131.

Figure 2. Recombinant HPTPbeta dephosphorylates recombinant VEGFR2 and Tie-2 kinases *in vitro*. (A) Graph showing increased substrate fluorescence with increasing amounts of recombinant HPTPbeta intracellular domain using the DiFMUP substrate. (B) Anti-phosphotyrosine western blots of recombinant GST fusions of the tyrosine kinase domains of the VEGFR2 and Tie-2 receptors showing a dose dependant decrease in phosphorylation after incubation with increasing amounts of recombinant HPTPbeta.

Figure 3. Overexpression of HPTPbeta using either a plasmid or adenoviral vector increases phosphatase activity in HEK293H cells and HUVEC's, respectively. (A) pCMV6-XL4/HPTPβ transfected (■) HEK293H cells have increased phosphatase activity over mock transfected (□) controls as measured with DiFMUP. (B) Increased phosphatase activity in HUVEC's infected with the HPTPbeta adenovirus (■) versus the green fluorescent protein (GFP) control adenovirus (□). The phosphatase activity in both cell types is inhibited with 500 μM of the nonselective tyrosine phosphatase inhibitor BMOV.

Figure 4. Transient overexpression of HPTPbeta attenuates ligand-induced autophosphorylation of VEGFR2 and Tie2 stably expressed in HEK293 cells. Phosphotyrosine

(P-Tyr), VEGFR2, Tie-2 and HPTPbeta western blots and graphs depicting a blunted ligand induced phosphorylation of the VEGFR2 receptor (A) and the Tie-2 receptor (B) in HEK293-receptor stable cells when transfected with the pCMV6-XL4/HPTPβ expression plasmid. (□ = mock transfected, ■ = HPTPbeta transfected, * = $p<0.05$)

Figure 5. Overexpression of HPTPbeta attenuates ligand-mediated autophosphorylation of endogenous VEGFR2 and Tie2 in human umbilical vein endothelial cells (HUVEC). (A) Anti-phosphotyrosine western blots of immunoprecipitated VEGFR2 from VEGF stimulated HUVEC's illustrating a decrease in the level of VEGFR2 phosphorylation when HPTPbeta is overexpressed and corresponding graph depicting cumulative VEGFR2 receptor activation data with and without HPTPbeta overexpression, n=3. (B) Anti-phosphotyrosine western blots of immunoprecipitated Tie-2 from Ang-1 stimulated HUVEC's showing a decrease in the level of Tie-2 receptor phosphorylation when HPTPbeta is overexpressed and a graph depicting cumulative data of Tie-2 receptor activation in empty and HPTPbeta adenovirus infected HUVEC's, n=3. (□ = uninfected, ■ = empty virus, ▣= HPTPbeta virus, * = $p<0.05$)

Figure 6. Overexpression of HPTPbeta in HUVEC attenuates VEGF-mediated calcium mobilization. Endothelial cells infected with either wild type or inactive HPTPbeta adenoviruses were loaded with the calcium sensing dye Fluo-4 (Molecular Probes) and challenged with increasing concentrations of recombinant $VEGF_{165}$. Graph shows a decrease in the magnitude of the $VEGF_{165}$ stimulated calcium flux in wild type HPTPbeta infected cells (▲) compared to uninfected controls (■). Conversely, an increase in VEGF stimulated calcium flux was seen in the cells infected with a catalytically inactive form of HPTPbeta (●) (catalytic Cysteine mutated to a Serine). No change in the EC_{50} values for $VEGF_{165}$ was observed within the treatment groups. Data represented as % of maximal calcium response to 10μM ionomycin.

Figure 7. Antisense "knockdown" of HPTPbeta expression enhances the activation of VEGFR2 and Tie2 in endothelial cells. (A) Western blots of VEGFR2 immunoprecipitates from VEGF stimulated HUVEC's shows a significant increase in the level of VEGFR2 phosphorylation when HPTPbeta protein levels are decreased by 60%. Graph depicts cumulative data from 3 experiments. (B) Western blots and graph showing increased Tie-2 phosphorylation in antisense oligo transfected cells (n=4). (□ = control oligo, ■ = HPTPbeta antisense oligo) (* = $p<0.05$).

Figure 8. Altering HPTPbeta expression in HUVECs using adenovirus overexpression or antisense knockdown modulates VEGF-mediated capillary morphogenesis in the endothelial

cell bead sprouting assay. (A) HPTPbeta overexpression decreases VEGF-induced sprouting. HPTPbeta and GFP infected HUVECs are incubated in the presence (■) or absence (□) of $VEGF_{165}$. (B) Antisense knockdown of HPTPbeta enhances VEGF induced endothelial cell sprouting.

Figure 9. Overexpression of HPTPbeta attenuates angiogenesis in the rat aortic ring explant assay. (A) Rat thoracic aortic rings are infected with empty virus, GFP containing virus or HPTPbeta containing virus and compared to uninfected rings. Representative images are shown for each group. (B) Quantitation of vessel extent and area indicate a significant reduction in both parameters only in HPTPbeta infected rings. Data are from 4-5 rings in each group. All viruses are administered at 1x1011 virus particles/ml. Abbreviations: Basal -Uninfected; EV - rings infected with an empty adenoviral vector; GFP - rings infected with adenovirus encoding green fluorescent protein; HPTPbeta - rings infected with adenovirus encoding HPTPbeta. * $P<0.001$ vs. Basal, GFP, EV, One-way ANOVA, followed by Tukey's Multiple Comparison Test.

Figure 10. Compound 1 ((R)-[1-Methylcarbamoyl-2-(4-sulfoamino-phenyl)-ethyl]-carbamic acid tert-butyl ester) is a selective HPTPbeta inhibitor. Compound 1 was assayed for inhibition of a panel of recombinant phosphatases essentially as described in Example 2 for HPTPbeta. This compound was at least 10 fold selective for HPTPbeta over the other phosphatases tested except for the closely related HPTPeta.

Figure 11. A selective HPTPbeta inhibitor enhances ligand-induced phosphorylation of VEGFR2 and Tie2. Endothelial cells pretreated for 30 minutes with either 0.1 mM (▣) or 1mM (■) Compound 1 show increased ligand stimulated tyrosine phosphorylation of the VEGFR2 (A) and Tie-2 (B) receptors compared to vehicle treated controls (□) as measured by anti-phosphotyrosine western blotting. * = $p<0.05$.

Figure 12. A selective HPTPbeta inhibitor enhances VEGF and Angiopoietin-1 (Ang-1) mediated endothelial cell survival. Endothelial cells treated with 500μM of the HPTPbeta inhibitor Compound 1 (■) show increased cell survival when stimulated with 500pM $VEGF_{165}$ or 250 ng/mL Ang-1 compared to vehicle treated controls (□).

Figure 13. A selective HPTPbeta inhibitor enhances angiogenesis in the rat aortic ring explant assay. Rat thoracic aortic rings were treated at the time of plating with increasing concentrations of a small molecule inhibitor of HPTPbeta. Representative pictures show increased sprouting in the rings treated with 100μM and 300μM Compound 1 (A). Graphed data from 8

quantitated pictures per treatment show significant increases in the parameters of vessel extent and area with 100μM and 300μM Compound 1 (B). * = $p<0.05$ vs. control.

SEQUENCE LISTING DESCRIPTION

Each of the nucleotide and protein sequences in the sequence listing, along with the corresponding Genbank or Derwent accession number(s) and animal species from which it is cloned, is shown in Table I.

Table I

Sequence Description	**SEQ ID NO: Nucleotide, Amino Acid**	**Species**	**Genbank (GB) or Derwent (D) Accession No. for Nucleotide Sequence**	**Related Genbank (GB) or Derwent (D) Accession Nos.**
HPTPβ (HPTP-Beta, PTPRB, PTPβ, PTPB, R-PTP-Beta)	1, 2	*Homo Sapiens*	X54131	NM_002837
mRPTPB (VE-PTP, R-PTP-Beta, mPTP-Beta)	3, 4	*Mus Musculus*	X58289	AF157628, XM_125813, Z23056
KDR (VEGFR2, Flk1)	5, 6	*Homo Sapiens*	AF063658	NM_002019
Tie-2 (TEK)	7, 8	*Homo Sapiens*	L06139	NM_000459, U53603
HPTPbeta intracellular domain	9	*Homo Sapiens*		
VEGFR2 Intracellular region containing kinase domain	10, 11	*Homo sapiens*	AF063658	
Tie-2 Intracellular region containing kinase domain	12, 13	*Homo sapiens*	L06139	
HPTPbeta variant	14, 15	*Homo sapiens*		
HPTPbeta crystallized domain	16	*Homo sapiens*		
HPTPbeta reverse primer	17	*Homo sapiens*		

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions.

Hereinafter, the use of the terms HPTPbeta, VEGFR2, and Tie-2 should be construed such that variants of these proteins are encompassed within the meaning of the term. For example, the term "HPTPbeta" should be construed as HPTPbeta and variants thereof. "Variants," as used herein, means variant sequences of those proteins, or nucleotide sequences encoding the same (all used herein interchangeably) that are substantially similar to those described for their wild type counterpart. A protein or nucleotide sequence may be altered in various ways to yield a variant encompassed by the present invention including substitutions, deletions, truncations, insertions, and modifications. Methods for such manipulations are well known in the art. For example, variants can be prepared by mutations in the nucleotide sequence. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Current Protocols in Molecular Biology (and updates) Ausubel et al. Eds. (1996), John Wiley and Sons, New York; Methods in Molecular Biology, vol 182, In vitro Mutagenesis Protocols, 2nd Edition, Barman Ed. (2002), Humana Press), and the references cited therein. In one embodiment, the substitution of the amino acid is conservative in that it minimally alters the biochemical properties of the variant. In other embodiments, the variant may be an active or inactive fragment of a full-length protein, a post-translationally or chemically modified protein, a protein modified by addition of affinity or epitope tags, or fluorescent or other labeling, whether accomplished by *in vivo* or *in vitro* enzymatic treatment of the protein, by chemical modification, or by the synthesis of the protein using modified amino acids. Non-limiting examples of modifications to amino acids include phosphorylation of tyrosine, serine, and threonine residues; glycosylation of serine, threonine, or asparagine residues; and ubiquitination of lysine residues.

In yet another embodiment, peptide mimics of a nucleic acid of the invention are encompassed within the meaning of variant. As used herein, "mimic," means an amino acid or an amino acid analog that has the same or similar functional characteristics of an amino acid. Examples of organic molecules that can be suitable mimics are listed at Table I of U.S. Pat. No. 5,807,819. Generally, a peptide variant, or nucleic acid sequence encoding the same, of the present invention will have at least 70%, generally, 80%, preferably up to 90%, more preferably 95%, even more preferably 97%, still even more preferably, and most preferably 99% sequence identity to its

respective native amino acid sequence. Fusion proteins, or N-terminal, C-terminal or internal extensions, deletions, or insertions into the peptide sequence shall not be construed as affecting homology.

"Sequence Identity" or "Homology" at the amino acid or nucleotide sequence level is determined by BLAST algorithms (Basic Local Alignment Search Tool) (Altschul et al., Nucleic Acids Res. 25, 3389-3402 (1997) which are tailored for sequence similarity searching. The default scoring matrix used by various BLAST algorithms is the BLOSUM62 matrix, Henikoff et al. Proc. Natl. Acad. Sci. USA 89, 10915-10919 (1992), recommended for query sequences over 85 nucleotides or amino acids in length.

"Isolated," as used herein, refers to nucleic acid or amino acid sequences that are removed from their natural environment and are isolated or separated, and are at least about 60% free, preferably about 75% free, and most preferably about 90% free from other components with which they are naturally associated.

The term "angiogenesis mediated disorder" is defined as a disorder that involves a modulation in angiogenic activity resulting in the biological manifestation of a disease, disorder, and/or condition; in the biological cascade leading to the disorder; or as a symptom of the disorder. The Applicants have shown that the process of angiogenesis is modulated by HPTPbeta. This "involvement" of HPTPbeta in an angiogenesis mediated disorder includes, but is not limited to, the following: (1) The modulation of HPTPbeta activity as a "cause" of the angiogenesis mediated disorder or biological manifestation, whether the HPTPbeta is modulated genetically, by infection, by autoimmunity, trauma, biomechanical causes, lifestyle, or by some other causes; (2) The modulated HPTPbeta activity is part of the observable manifestation of the disease or disorder. That is, the disease or disorder is measurable in terms of the modulated HPTPbeta activity. From a clinical standpoint, modulated HPTPbeta activity indicates the disease, however, HPTPbeta activity need not be the "hallmark" of the disease or disorder; (3) The modulated HPTPbeta activity is part of the biochemical or cellular cascade that results in the disease or disorder. In this respect, inhibiting or stimulating of HPTPbeta (per the respective therapeutic goal) interrupts the cascade, and thus controls the disease; (4) The angiogenesis mediated disease or disorder is not the direct result of modulation in HPTPbeta activity per se, but modulation of the HPTPbeta activity would result in amelioration of the disease. "Modulation in HPTPbeta activity," as used herein, encompasses both unwanted or elevated HPTPbeta activity and desired or reduced HPTPbeta activity. As used herein,

"angiogenesis mediated disorders" include: (1) those disorders, diseases and/or unwanted conditions which are characterized by unwanted or elevated angiogenesis, or (2) those disorders, diseases and/or unwanted conditions which are characterized by wanted or reduced angiogenesis."

"Protein," is used herein interchangeably with peptide and polypeptide.

II. Methods of identifying an agent useful for modulating angiogenesis.

The present invention is based, in part, on the discovery that modulation of HPTPbeta activity will modulate the activity of VEGFR2 and Tie-2. Applicants' studies demonstrate that a truncated version of recombinant HPTPbeta phosphatase domain dephosphorylates both Tie-2 and VEGFR2. Applicants' studies also demonstrate that full-length HPTPbeta attenuates the ligand-mediated activation of both Tie-2 by Ang1, and of VEGFR2 by VEGF. Further studies demonstrate the transfection of human endothelial cells with HPTPbeta antisense oligonucleotides results in about a 60% reduction of HPTPbeta expression and an enhanced ligand-mediated activation of both Tie-2 and VEGFR2. Applicants' studies demonstrate that overexpression of HPTPbeta inhibits angiogenesis in a VEGF-dependent *ex vivo* model of blood vessel growth (rat aortic ring model). Conversely, Applicants show that "knocking down" endogenous HPTPbeta expression using antisense oligos in endothelial cells enhances VEGF-mediated capillary morphogenesis. In view of the foregoing, Applicants demonstrate that HPTPbeta is a useful target in identifying agents for the treatment of angiogenesis mediated disorders.

Without limitation, the assays of the present invention are useful for high throughput screening or cell based assay formats.

1. Nucleic Acids of The Invention

The present invention further provides recombinant DNA (rDNA) molecules that contain a coding sequence of or a variant form of the molecules of invention. Methods for generating recombinant DNA molecules are well known in the art; for example, see Ausubel et al. (1996) Current Protocols in Molecular Biology John Wiley & Sons; Sambrook *et al.* (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory Press. In the preferred rDNA molecules, a coding DNA sequence is operably linked to expression control sequences and/or vector sequences.

Various commercially available vectors may be employed to clone the genes of the invention that may serve different purposes. Vectors of the present invention may be capable of directing the replication either in a prokaryotic or eukaryotic host, insertion into the host chromosome, or expression of the gene. Control elements include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, enhancers, and other regulatory elements. Preferably, the inducible promoter is readily controlled, such as being responsive to a nutrient or antibiotic in the medium.

2. Host Cells Expressing Nucleic Acids

Cells that naturally express nucleic acids and proteins of the invention may be known in the art. Cell lines that comprise enhanced levels of nucleic acids and proteins of the invention may be either purchased commercially or constructed. The present invention further provides host cells transformed with a nucleic acid molecule that encode sequences listed in Table 1 or their variants. The host cell can be either a prokaryotic or a eukaryotic cell. Preferred eukaryotic host cells include yeast, insect, Chinese hamster ovary (CHO), Swiss or NIH 3T3, baby hamster kidney (BHK), and the like.

Transformation of appropriate cell hosts with a recombinant DNA molecule of the present invention is accomplished by well-known methods that typically depend on the type of vector used and host system employed. With regard to transformation of prokaryotic host cells, heat shock methods, electroporation and salt treatment methods can be employed. With regard to transfection of vertebrate cells electroporation, cationic lipids, calcium phosphate precipitation, gene gun, microinjection, or protoplast fusion methods can be employed (See Ausubel et al. (1996) supra).

Successfully transformed cells, *i.e.*, cells that contain an rDNA molecule of the present invention, can be identified by well known techniques including the selection for a selectable marker. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence or expression of the rDNA using Southern, Northern, or Western blots, or the proteins produced from the cell may be assayed via an immunological method.

3. Proteins of the Invention

A protein of the invention may be obtained by methods well known in the art, for example, using standard direct peptide synthesizing techniques (e.g., as summarized in Bodanszky, Principles of Peptide Synthesis, Springer-Verlag, Heidelberg: (1984)), such as via solid-phase synthesis (see, e.g., Merrifield, J. Am. Chem. Soc., 85, 2149-54 (1963); Barany et al., Int. J. Peptide Protein Res., 30, 705-739 (1987); and U.S. Pat. No. 5,424,398). If the gene sequence is known or can be deduced from the polypeptide sequence then the protein may be produced by standard recombinant methods. The proteins may be isolated or purified in a variety of ways known to those skilled in the art. Standard purification methods include precipitation with salts, electrophoretic, and chromatographic techniques, including ion exchange, hydrophobic, affinity, and reverse-phase HPLC chromatography, chromatofocussing, and others (see, e.g., Scopes, Protein Purification: Principles and Practice (1982); U.S. Pat. No. 4,673,641; Ausubel et al. supra; and Sambrook et al, supra). Ultrafiltration and diafiltration techniques, in conjunction with protein concentration, are also useful.

Antibody probes to the proteins of the invention can be prepared by immunizing suitable mammalian hosts utilizing appropriate immunization protocols using the proteins of the invention or antigenic fragments thereof. While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, use of monoclonal preparations is preferred. Immortalized cell lines which secrete the desired monoclonal antibodies may be prepared using standard methods, (see e.g., Kohler & Milstein, Biotechnology, 24, 524-526 (1992) or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known.

Fragments of the monoclonal antibodies or the polyclonal antisera that contain the immunologically significant portion can be used as agonist or antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as Fab or Fab' fragments, is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin.

The antibodies or fragments may also be produced, using current technology, by recombinant means. Antibody regions that bind specifically to the desired regions of the protein can also be produced in the context of chimeras with multiple species origin, for instance, humanized antibodies. The antibody can therefore be a humanized antibody or human antibody, as described in U.S. Patent 5,585,089 or Riechmann et al., Nature 332, 323-327 (1988).

4. Selection of Test Agents

Agents that can be screened in accordance with the assays of the invention include but are not limited to, libraries of known agents, including natural products, such as plant or animal extracts, synthetic chemicals, biologically active materials including proteins, peptides including but not limited to members of random peptide libraries and combinatorial chemistry derived molecular library made of D- or L- configuration amino acids, phosphopeptides (including, but not limited to, members of random or partially degenerate, directed phosphopeptide libraries), antibodies (including, but not limited to, polyclonal, monoclonal, chimeric, human, anti-idiotypic or single chain antibodies, and Fab, $F(ab')_2$ and Fab expression library fragments, and epitope-binding fragments thereof), and organic and inorganic molecules.

In one embodiment an agent that may modulate expression of a gene of the invention is a polynucleotide. The polynucleotide may be an antisense, a small interference RNA (siRNA), a triplex agent, or a ribozyme. For example, an antisense may be directed to the structural gene region or to the promoter region of a gene of the invention.

In addition to the more traditional sources of test agents, computer modeling and searching technologies permit the rational selection of test agents by utilizing structural information from both the ligand binding sites of proteins of the present invention and their known ligands. Such rational selection of test agents can decrease the number of test agents that must be screened in order to identify a candidate therapeutic agent. Knowledge of the sequences of proteins of the present invention allows for the generation of models of their binding sites that can be used to screen for potential ligands. This may be accomplished using molecular modeling with either the known crystal- and NMR-based three-dimensional structures of the proteins of the present invention. General information regarding modeling can be found in Schoneberg, T. et. al., *Molecular and Cellular Endocrinology,* 151:181-193 (1999), Flower, D., *Biochimica et Biophysica Acta,* 1422:207-234 (1999), and Sexton, P.M., *Current Opinion in Drug Discovery and Development,* 2(5):440-448 (1999).

Once the model is completed, it can be used in conjunction with one of several existing computer programs to narrow the number of agents to be screened by the screening methods of the present invention, like the DOCK program (UCSF Molecular Design Institute, 533 Parnassus Ave, U-64, Box 0446, San Francisco, California 94143-0446). In several of its variants it can screen databases of commercial and/or proprietary agents for steric fit and rough electrostatic

complementarity to the binding site. Another program that can be used is FLEXX (Tripos Inc., 1699 South Hanley Rd., St. Louis, MO).

5. In vitro Screening Assays to Identify Candidate Agents

The finding that the genes of the present invention play a role in regulating angiogenesis enables various methods of screening one or more test agents to identify candidate agents that ultimately may be used for prophylactic or therapeutic treatment. The invention provides methods for screening test agents for their ability, inter alia, to bind to a protein of the present invention, activate a protein of the present invention, prolong or augment the agonist-induced activation of a protein of the present invention or of signal transduction pathways that regulate expression of a gene(s) of the present invention.

When selecting candidate agents, it is preferable that the candidate agents be selective for a protein(s) of the invention. "Selective" means that the agent has significantly greater activity toward a certain protein(s) compared with other proteins, not that it is completely inactive with regard to other receptors. A selective agent for a specific receptor may show 10-fold, preferably 100-fold, more preferably 1000-fold and most preferably greater than 1000-fold selectivity toward that protein of the present invention than other related or unrelated proteins. Therefore, the purpose here is to identify and select an agent that would show minimal cross-reactivity and therefore minimal side effects when administered to an animal, but it should be kept in mind that other homologous proteins may show limited cross-reactivity. For screening agents it is preferred that the initial *in vitro* screen be carried out using a protein of the invention with an amino acid sequence that is greater than 80% homologous to a sequence listed in the sequence listing. More preferably the test agents will be screened against a human, or mouse protein, with the most preferable being human. For screening agents in a non-human species it is preferable to use the protein from the species in which treatment is contemplated.

The methods of the present invention are amenable to high throughput applications; however, the use of as few as one test agent in the method is encompassed by the term "screening". Test agents which bind to a protein of the invention, activate a protein of the invention, prolong or augment the agonist-induced activation of a protein of the invention or its signal transduction pathway, or increase expression of a protein or a gene of the invention, as determined by a method of the present invention, are referred to herein as "candidate agents." Such candidate agents can be

used to regulate angiogenesis mediated disorders. However, more typically, this first level of *in vitro* screen provides a means by which to select a narrower range of agents, i.e., the candidate agents, which merit further investigation at additional levels of screening. The skilled artisan will recognize that a utility of the present invention is to identify, from a group of one or more test agents, a subset of agents which merit further investigation. One of skill in the art will also recognize that the assays of the present invention are useful in ranking the probable usefulness of a particular candidate agent relative to other candidate agents. Using such information the skilled artisan may select a subset of the candidate agents, identified in the first level of screening, for further investigation. By the way of example only, agents which activate a protein of the invention at concentrations of less than 200 nM might be further tested in a different assay system that may involve use of organ culture or in vivo models of angiogenesis.

The assay systems described below may be formulated into kits comprising a protein of the invention or cells expressing a protein of the invention which can be packaged in a variety of containers, e.g., vials, tubes microtitre well plates, bottles and the like. Other reagents may be included in separate containers and provided with the kit, e.g., positive and negative control samples, buffers, etc.

In one embodiment, the invention provides a method for screening one or more test agents to identify candidate agents that bind to a protein of the invention. Methods to determine binding of an agent to a protein are well known in the art. Typically, the assays include the steps of incubating a source of a protein of the invention with a labeled agent, known to bind to the protein, in the presence or absence of a test agent and determining the amount of bound labeled agent. The source of a protein of the invention may either be cells expressing the protein or some form of isolated protein, as described herein. The labeled agent can be a known ligand or any ligand analog labeled such that it can be measured, preferably quantitatively (e.g., ^{125}I-, europium-, fluorescein-, GFP-, or ^{35}S-methionine labeled). Such methods of labeling are well known in the art. Test agents that bind to a protein of the invention cause a reduction in the amount of labeled ligand bound to the protein, thereby reducing the signal level compared to that from control samples (absence of test agent). Variations of this technique have been described in literature. See Keen, M., *Radioligand Binding Methods for Membrane Preparations and Intact cells* in Receptor Signal Transduction Protocols, R.A.J. Challis, (ed), Humana Press Inc., Totoway N.J. (1997).

In another embodiment, the invention provides methods for screening test agents to identify candidate agents that activate a protein of the invention. Typically, the assays are cell-based; however, cell-free assays are known which are able to differentiate agonist and antagonist binding. Cell-based assays include the steps of contacting cells which express a protein of the invention with a test agent or control and measuring activation of the protein by measuring the expression or activity of components of the affected signal transduction pathways. For example, after contact with the test agent, lysates of the cells can be prepared and assayed for induction of second messengers like free intracellular calcium concentration [Ca^{2+}], cAMP, or phosphorylation levels of a downstream signaling protein or of the protein itself if the protein is a kinase or phosphatase, or activity of the downstream signaling molecules.

For example, intracellular free calcium concentrations are measured using techniques well known in the art. Cells are loaded with a suitable fluorescent dye, like Fura-2 or FLUO-4 (Molecular Probes), and changes in [Ca^{2+}] in response to different stimuli are detected using either a fluorescent microscope or a fluorescent plate reader. Ionophores like, Ionomycin may be used to measure the total [Ca^{2+}] in a cell.

Similarly, cAMP induction is measured with the use of DNA constructs containing the cAMP responsive element linked to any of a variety of reporter genes can be introduced into cells expressing a protein of the invention. Such reporter genes include, but are not limited to, chloramphenicol acetyltransferase (CAT), luciferase, glucuronide synthetase, growth hormone, fluorescent proteins, or alkaline phosphatase. Following exposure of the cells to the test agent, the level of reporter gene expression can be quantified to determine the test agent's ability to increase cAMP levels and thus determine a test agent's ability to activate a protein of the invention.

The activity of a phosphatase can be measured by methods well known in the art (Wang Y, Journal of Biological Chemistry, 267, 16696, 1992; Harder Kwet al., Biochemistry Journal, 298, 395, 1994; Itoh et al., Journal of Biological Chemistry, 267, 12356, 1992). In one format, the phosphatase activity is measured using a fluorescent assay that generates a fluorescent signal when the substrate is acted upon by the enzyme. Other small molecule phosphatase substrates such as PNPP (para nitro phenyl phosphate) could also be used. These assay formats may be scaled-up for utilization in a high throughput screening assays using FRET (fluorescence resonance energy transfer) FP (Fluorescence polarization) or Malachite green assay. Another means of assaying for phosphatase activity is to measure the loss of phosphorylation of its known substrates or receptor tyrosine kinase domain

fusion proteins or synthetic peptides carrying phosphotyrosine residues. A phosphotyrosine western blot may be used to determine the decrease in phosphotyrosine content of a target protein in a suitable assay format (Huang et al., J Biol Chem 274, 38183-38188, 1999).

In another embodiment, specific phospho-tyrosine or phospho-serine antibodies may be utilized to measure the level of phosphorylation of a signaling protein after the exposure to a test agent, whereby the significant deviation in phosphorylation levels compared to control samples would indicate activation of a protein of the invention. The activity of a substrate may be measured by methods well known in the art. In one embodiment, the substrate activity is measured by assaying with anti-phosphotyrosine Western blotting following immunoprecipitation of the substrate, or ELISA like KIRA (Kinase-Inhibitor Receptor Assay). If a biological assay is known for the substrate it may also be employed, like an angiogenesis assay.

In some instances, a protein's (for example receptor) responses subside, or become desensitized, after prolonged exposure to an agonist. Another embodiment of the invention provides methods for identifying agents that prolong or augment the agonist-induced activation of a protein of the invention, or the activation of signal transduction pathway, in response to an agonist of a protein of the invention. Such agents may be used, for example, in conjunction with an agonist. Typically the method uses a cell based assay comprising: i) contacting a test agent with a first cell population which expresses a functional protein of the invention; ii) treating a second cell population with an agonist for the protein of the invention for a sufficient time and at a sufficient concentration to cause desensitization of the protein; iii) further treating the second cell population with the test agent; iv) determining the level of activation of the protein in the first and second cell population; and v)identifying those test agents that prolong or augment the activation of the protein of the invention or its signal transduction pathway as candidate agents. One of skill in the art will recognize that several mechanisms contribute to protein desensitization including, but not limited to, protein phosphorylation, protein internalization or degradation and signal transduction pathway down-modulation. One of skill in the art can determine the appropriate time (i.e., before, during or after agonist treatment) for contacting the cells with the test agents depending upon which mechanism of desensitization is targeted. For example, contacting the cells with test agents following agonist treatment, can detect test agents which block protein desensitization which occurs as a result of phosphorylation of the protein.

In another embodiment, the invention provides a method of screening one or more test agent to identify candidate agents that regulate transcription of the gene or regulate expression of the protein of the invention. Candidate agents that regulate transcriptional activity of genes of the invention may be identified using a reporter gene operably associated with the regulatory region of the gene of the invention (reporter gene construct). Such methods are known in the art. In one such method, the reporter gene construct is contacted with a test agent in the presence of a source of cellular factors and the level of reporter gene expression is determined. A test agent that causes an increase in the level of expression, compared to a control sample, is indicative of a candidate agent that increases transcription of the gene. To provide the cellular factors required for *in vitro* or *in vivo* transcription, appropriate cells or cell extracts are prepared from any cell type that normally expresses the gene.

Candidate agents that regulate expression of a gene of the invention can also be identified by a method wherein a cell is contacted with a test agent and the expression of the gene is determined. The level of expression of the gene in the presence of the test agent is compared with the level of expression in the absence of the test agent. Test agents that increase the expression are identified as candidate agents. Such a method detects candidate agents which increase the transcription or translation of the gene or which increase the stability of the mRNA or protein.

Additional assay formats may be used to monitor the ability of an agent to modulate the expression of a gene. For instance, mRNA expression may be monitored directly by hybridization. Other means to evaluate expression levels is to use either quantitative or semi-quantitative PCR or RNAse Protection Assay (Ma et al., Methods 10, 273-238 (1996). An example of quantitative PCR is the use of TaqMan™ analysis developed and described by Applied Biosystems, (ABI).

6. Screening Assays using in vitro and in vivo models of angiogenesis

Candidate agents selected on the basis of modulating phosphatase activity directly against a phosphatase or indirectly in cell based assays described above may then be further screened in angiogenesis assays that are well known in the art. Such assays include *in vitro* assays that measure surrogates of blood vessel growth in cultured cells or formation of vascular structures from tissue explants and *in vivo* assays that measure blood vessel growth directly or indirectly (Auerbach,R., et al. (2003). Clin Chem 49, 32-40, Vailhe,B., et al. (2001). Lab Invest 81, 439-452).

a. In vitro models of angiogenesis

Most of these assays employ cultured endothelial cells or tissue explants and measure the effect of candidate agents on "angiogenic" cell responses or on the formation of blood capillary-like structures. Examples of *in vitro* angiogenesis assays include but are not limited to endothelial cell migration and proliferation, capillary tube formation, endothelial sprouting, the aortic ring explant assay and the chick aortic arch assay.

b. In vivo models of angiogenesis

In these assays candidate agents are administered locally or systemically in the presence or absence of growth factors (i.e. VEGF or angiopoietin 1) and new blood vessel growth is measured by direct observation or by measuring a surrogate marker such as hemoglobin content or a fluorescent indicator. Examples of angiogenesis include but are not limited to chick chorioallantoic membrane assay, the corneal angiogenesis assay, and the matrigel plug assay.

III. Treatment of angiogenesis mediated disorders

1. Treatment of disorders mediated by elevated angiogenesis

As previously described, an angiogenesis mediated disorder encompasses disorders resulting from either elevated or reduced angiogenesis. The agents screened by the present invention may be used in a method for the treatment of a disorder mediated by elevated angiogenesis. Such agents identified by the present invention may be used to treat diseases like diabetic retinopathy, macular degeneration, sickle cell anemia, sarcoid, syphilis, pseudoxanthoma elasticum, Paget's disease, vein or artery occlusion, carotid obstructive disease, chronic uveitis/vitritis, mycobacterial infections, Lyme's disease, systemic lupus erythematosis, retinopathy of prematurity, Eales' disease, Behcet's disease, infections causing retinitis or choroiditis, presumed ocular histoplasmosis, Best's disease, myopia, optic pits, Stargardt's disease, pars planitis, chronic retinal detachment, hyperviscosity syndromes, toxoplasmosis, trauma, post-laser complications, diseases associated with rubeosis (neovascularization of the angle) and diseases caused by the abnormal proliferation of fibrovascular or fibrous tissue including all forms of proliferative vitreoretinopathy. Agents screened by of the present invention can also treat diseases associated with chronic inflammation such as Crohn's disease and ulcerative colitis, psoriasis, sarcoidosis and rheumatoid arthritis. Other diseases that can be treated according to the present invention are hemangiomas, Osler-Weber-Rendu disease, or

hereditary hemorrhagic telangiectasia, solid or blood borne tumors and acquired immune deficiency syndrome.

2. Treatment of disorders mediated by reduced or limited angiogenesis

In one aspect, an agent may be used in a method for the treatment of a disorder mediated by reduced angiogenesis. A disorder mediated by reduced angiogenesis is one characterized by a tissue that is suffering from or is at risk of suffering from ischemic damage, infection, and/or poor healing, which results when the tissue is deprived of an adequate supply of oxygenated blood due to inadequate circulation (ischemic tissue). Non-limiting examples of angiogenesis reduced disorders that may be treated by the present invention are herein described below.

a. Method of vascularizing ischemic tissue

In one aspect, an agent that modulates HPTPbeta activity may be used in a method of vascularizing ischemic tissue. Examples of ischemic tissue include, but are not limited to, tissue that lack adequate blood supply resulting from myocardial and cerebral infarctions, mesenteric or limb ischemia, or the result of a vascular occlusion or stenosis. In one example, the interruption of the supply of oxygenated blood may be caused by a vascular occlusion. Such vascular occlusion can be caused by arteriosclerosis, trauma, surgical procedures, disease, and/or other indications. There are many ways to determine if a tissue is at risk of suffering ischemic damage from undesirable vascular occlusion. For example, in myocardial disease these methods include a variety of imaging techniques (e.g., radiotracer methodologies, x-ray, and MRI) and physiological tests. Therefore, induction of angiogenesis in tissue affected by or at risk of being affected by a vascular occlusion is an effective means of preventing and/or attenuating ischemia in such tissue. Thus, the treatment of skeletal muscle and myocardial ischemia, stroke, coronary artery disease, peripheral vascular disease are fully contemplated.

b. Method of repairing tissue by enhancing limited angiogenesis

In another aspect, an agent that modulates HPTPbeta activity may be used in a method of repairing tissue. As used herein, "repairing tissue," means promoting tissue repair, regeneration, growth, and/or maintenance including, but not limited to, wound repair or tissue engineering. One skilled in the art readily appreciates that new blood vessel formation is required for tissue repair.

Tissue may be damaged by traumatic injuries or conditions including arthritis, osteoporosis, and other skeletal disorders, from injuries due to surgical procedures, irradiation, laceration, toxic chemicals, viral infection, bacterial infection, non-healing wounds, or burns.

Agents that modulate HPTPbeta activity may also be used in a method to aid in tissue repair in the context of guided tissue regeneration (GTR) procedures, like tissue engineering. Thus, the present method can be used to augment the design and growth of human tissues outside the body for later implantation in the repair or replacement of diseased tissues. For example, an agent that modulates HPTPbeta activity may be useful in promoting the growth of skin graft replacements that are used as a therapy in the treatment of burns.

In another aspect of tissue engineering, an agent that modulates HPTPbeta activity may be included in cell-containing or cell-free devices that induce the regeneration of functional human tissues when implanted at a site that requires regeneration. As previously discussed, biomaterial-guided tissue regeneration can be used to promote bone regrowth in, for example, periodontal disease. Thus, an agent that modulates HPTPbeta activity may be used to promote the growth of reconstituted tissues assembled into three-dimensional configurations at the site of a wound or other tissue in need of such repair.

In another aspect of tissue engineering, an agent that modulates HPTPbeta activity can be included in external or internal devices containing human tissues designed to replace the function of diseased internal tissues. This approach involves isolating cells from the body, placing them on or within structural matrices, and implanting the new system inside the body or using the system outside the body. The method of the invention can be included in such matrices to promote the growth of tissues contained in the matrices. For example, an agent that modulates HPTPbeta activity can be included in a cell-lined vascular graft to promote the growth of the cells contained in the graft. It is envisioned that the method of the invention can be used to augment tissue repair, regeneration, and engineering in products such as cartilage and bone, central nervous system tissues, muscle, liver, gastrointestinal tract (includes IBD and ulcer diseases) and pancreatic islet (insulin-producing) cells.

IV. Diagnostic or Prognostic Methods

Expression of HPTPbeta or nucleic acids encoding the same may be used as a diagnostic marker for the prediction or identification of an angiogenesis mediated disorder. For example, a cell or tissue sample may be assayed for the expression levels of HPTPbeta by any of the methods

described herein and compared to the expression level found in normal healthy tissue. Such methods may be used to diagnose or identify angiogenesis mediated disorders.

Expression of HPTPbeta or nucleic acids encoding the same may also be used as a marker for the monitoring of the progression of an angiogenesis mediated disorder. Expression or activity of HPTPbeta or nucleotides encoding the same may also used to track or predict the progress or efficacy of a treatment regime in a patient. For instance, a patient's progress or response to a given drug may be monitored by measuring gene expression of HPTPbeta of the invention in a cell or tissue sample after treatment or administration of the drug. The expression of HPTPbeta in the post-treatment sample may then be compared to gene expression from the same patient before treatment.

EXAMPLES

Example 1. Isolation of a full length HPTPbeta cDNA clone

Methods: A full length clone of HPTPbeta is obtained by tiered screening of a human placenta arrayed cDNA library from Origene using the forward vector primer supplied and a reverse primer specific for HPTPbeta (5'-GTTAGGGAAGTAAATCGATACTGGG-3'). Polymerase chain reactions (PCR) are accomplished using Advantage Polymerase (Clontech) using the following temperature cycles: 1 cycle at 95 °C for 3 min, 30 cycles at 94 °C for 1 min and 62 °C for 2 min followed by 1 cycle at 62 °C for 3 min. According to the manufacturers instructions, bacterial sub-plates of positive cDNA pools are screened using the same primers and the subsequent positive bacterial colony containing a full length HPTPbeta cDNA (SEQ ID NO: 14) clone was isolated. Plasmid DNA are then isolated and sequenced.

Results: The isolated clone encoded a full length HPTPbeta protein (SEQ ID NO: 15), and has a stop codon at 3 prime end of the coding region. Sequence alignments showed that it was essentially identical to a previously published HPTPbeta clone (Genbank accession # X54131) except for the absence of 270 nucleotides corresponding to exon 6 suggesting that the clone encoded a novel splice variant. Confirming this impression, further review of the 5' end of the HPTPbeta gene revealed that the missing exon codes for FNIII (fibronectin like domain 3) repeat 5 (one of a total of 16 possible) (Figure 1).

Example 2. Recombinant HPTPbeta dephosphorylates recombinant VEGFR2 and Tie-2 kinases *in vitro*

Methods: Expression and purification of recombinant HPTPbeta catalytic domain is accomplished by subcloning the intracellular domain of HPTPbeta (cDNA clone starting at the codon for amino acid 1662 through the stop codon) into the pGEX-4T1 vector (Pharmacia) and expressed as a GST-fusion protein in *E.coli* BL21-RIL cells (Stratagene). Induced cells are thawed on ice and resuspended in 20mM Tris-HCl, pH=8.0, 1% Trition x-100, 2mM DTT and EDTA-free protease inhibitor cocktail. 10mg/ml lysozyme is added and cells are lysed by sonication. The cell debris is pelleted using the Beckman J2-MI at 17,000 rpm. The supernatant is added to prepared glutathione sepharose 4B resin batch method. After binding, the column is poured and packed with the resin/protein mixture and then washed. The column is unpacked for cleavage of GST using thrombin (50 units thrombin/1mL resin). Mixture is rotated O/N at room temperature. The column is re-packed for washing cleaved material from resin. Cleaved material is collected and then loaded onto Q sepharose FF column. The column is washed and protein eluted with 0-1M NaCl gradient. A coomassie blue stained SDS-PAGE gel identified fractions containing recombinant material and pooled fractions and dialyzed against 20mM Tris-HCl, pH 8.0, 10mM BME, 2mM DTT.

Measurements of recombinant phosphatase activity are performed using the substrate DiFMUP (6,8-difluoro-4-methylumbelliferyl phosphate) from Molecular Probes. 10μM DiFMUP is incubated with increasing amounts of recombinant HPTPbeta in buffer containing 10 mM Sodium Acetate (pH 6), 150 mM NaCl, 0.1%BSA, 5 mM DTT. Measurements of increased substrate fluorescence, indicative of phosphatase activity, are observed at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a Victor V plate reader (Wallac).

To prepare recombinant GST fusion receptor tyrosine kinase domains for kinase domain dephosphorylation assays, intracellular kinase domain cDNAs of the VEGFR2 and Tie-2 receptors are generated by PCR starting at the codon for the 1st amino acid inside the transmembrane domain, a lysine in both receptors, using forward primers having an "in-frame" Sal I site and reverse primer containing a Not I site for both receptors. The pLNCX2 plasmids below are used as templates. PCR fragments are subcloned into the GST fusion vector pACGHLT-A (Pharmingen/BD Biosciences) and sequenced through on both strands. Confirmed clones are transfected into sf9 insect cells using the BaculoGold™ system from Pharmingen. Resulting baculovirus stocks are amplified and final protein production is rendered in infected sf9 cells on 150mm dishes. Infected sf9 cells were lysed in 2mL of Triton Lysis Buffer (TLB, 20mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Triton X-

100, 2 mM EDTA, 1mM Sodium Orthovanadate, 1mM Sodium Fluoride, 1 mM PMSF, 1 ug/ml leupeptin, 1 ug/ml pepstatin), scraped, and frozen at –80°C until used.

Purification of recombinant GST fusion kinase domains is accomplished using GST agarose beads (Pharmacia) prepared according the package insert. Briefly, 100 uL of the infected sf9 lysate is mixed with 50 uL of washed GST agarose beads in 1mL TLB in batch form. After 4 hours, the beads are spun and washed twice in cold TLB. To ensure maximal phosphorylation of the recombinants, the beads are washed once and resuspended in 100uL of kinase buffer (200mM Tris-HCl, pH=8.0, 1M NaCl, 120 mM MgCl2, 10 mM DTT) including 1mM ATP and incubated for 30 minutes at room temperature. The beads, bound with phosphorylated recombinant kinase domains, are washed twice in assay buffer (10 mM TrisHCl pH = 7, 150 mM NaCl, 0.1% BSA, 5 mM DTT) and kinase domain dephosphorylation assays are performed by incubating the beads for 2 hours at 37°C with increasing amounts of recombinant HPTPbeta. After incubation, beads are rinsed twice with TLB and eluted by boiling for 10' in 1x SDS sample buffer. Samples are loaded on a 10% SDS-PAGE and transferred to PVDF for anti-phosphotyrosine western blotting (see example 4).

Results: Increased substrate fluorescence indicative of phosphatase activity is seen with increasing amounts of recombinant HPTPbeta intracellular domain using the DiFMUP substrate (Figure 2A). This recombinant HPTPbeta is able to dephosphorylate recombinant autophosphorylated kinase domains as seen by anti-phosphotyrosine western blotting which shows a dose dependant decrease in the phosphorylation of recombinant VEGFR2 and Tie-2 (Figure 2B.). These results demonstrate a method that could be used to screen for agents that inhibit HPTPbeta activity.

Example 3. HPTPbeta attenuates ligand induced VEGFR2 and Tie-2 receptor phosphorylation in cells overexpressing HPTPbeta.

Methods: HEK293 cells are transfected with the cDNA construct isolated from the Origene library, already in the mammalian expression vector pCMV6-XL4, using LipofectAMINE-PLUS (Invitrogen).

For expression in human umbilical vein endothelial cells (HUVEC's) adenoviruses are prepared and purified containing no insert (empty), full length HPTPbeta and green fluorescent protein (GFP) by Galapagos Genomics NV as described in US Patents: US 5,994,128 & US 6,033,908.

In cell phosphatase assays are accomplished by infecting Human Umbilical Vein Endothelial Cells with HPTPbeta, GFP, or Empty adenovirus in 100 mm dishes for 24 hours. Infected cells were trypsinized and re-plated at 5,000 cells/well in a 96 well plate and allowed to attach for 2 hours in complete growth medium (EGM). Complete medium was replaced with Hanks Buffered Saline (HBSS, Gibco) and substrate fluorescence are measured 15 minutes after the addition of 100uM DiFMUP substrate (Molecular Probes) using a Victor V plate reader as mentioned in Example 2.

For phosphatase activity in human embryonic kidney cells, HEK293H (Gibco) are grown in suspension in 293SFM media (Gibco) and transfected on 100 mm dishes using Lipofectamine 2000 (Invitrogen). After 24 hours cells are resuspended transferred back to shaker flasks for an additional 24 hours. Phosphatase activity is measured using DiFMUP in 96 well plates as above with 100,000 cells/well. For inhibition of tyrosine phosphatases, the cells are pre-incubated with Bis-maloalto-organovanadium (BMOV) for 15 minutes prior to the addition of DIFMUP. HPTPbeta protein expression is confirmed after each experiment in parallel plates by western blotting.

To establish stable cell lines expressing endothelial cell receptor tyrosine kinases, HEK293 cells are transfected with mammalian expression plasmids (pLNCX2, Clontech) containing either the full length human VEGF receptor type 2 (VEGFR2) cDNA or full length human Tie-2 receptor cDNA using LipofectAMINE-Plus (Invitrogen) on 100mm dishes. Forty-eight hours post transfection the cells are selected in growth medium (DMEM, 10% fetal bovine serum) supplemented with 1.4 mg/mL Geneticin™ (Gibco) (the concentration of Geneticin™ was determined by kill curve analysis of wild type HEK293 cells). After selection, isolated colonies are chosen using cloning cyclinders and the clonal cell lines propagated in complete selection media. Clonal cell lines were screened for the presence of the receptors by western blot as well as by receptor activation assay. The Tie-2 stable cells are designated T2-3 and the VEGFR2 stable cells designated R2-6.

VEGFR2 and Tie-2 receptor phosphorylation is assayed by anti-phosphotyrosine western blotting following immunoprecipitation of the receptors from endothelial cells or receptor transfected 293 cells. Briefly, endothelial cells (HUVEC's) are plated on 100mm dishes, stimulated with a dose range of either recombinant VEGF165 or recombinant Angiopoietin-1 (R&D Systems, resuspended in PBS containing 0.2% bovine serum albumin) for 5 or 7 minutes, respectively. After stimulation the cells were lysed in 1mL of Triton Lysis Buffer (TLB, 20mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1mM Sodium Orthovanadate, 1mM Sodium Fluoride,

1 mM PMSF, 1 ug/ml leupeptin, 1 ug/ml pepstatin) at 4 deg C. To immunoprecipitate VEGFR2, 1ml of the lysate from cells stimulated with VEGF165 is incubated with 25uL wheat germ agglutinin-agarose (Sigma, lectin from triticum vulgaris). Lysates stimulated with Ang-1 are immunoprecipitated using 2ug of anti Tie-2 antibody (Ab33) and 25uL of protein A/G Plus (Santa Cruz Biotechnology). Following overnight incubation, the complexes are centrifuged, washed once in TLB and eluted by boiling in 30uL of 1X sample buffer (50mM Tris-HCl pH=6.8, 10% glycerol, 2% SDS, 0.1 M DTT, 0.1% bromphenol blue). Twenty microliters of each sample is loaded onto a 6% SDS-PAGE gel, transferred to PVDF (Novex), blocked in 5% bovine serum albumin in TBS-0.1% Tween-20 (TTBS) and phosphotyrosine western blotting is performed using anti-phosphotyrosine antibody (PY99, Santa Cruz Biotechnology) diluted 1:1000 in 2.5% bovine serum albumin in TTBS. Signal is detected using HRP labeled secondary antibodies and ECL solution (Amersham). After exposure the blots are stipped and re-probed with anti-Tie-2 receptor (Ab33) or anti VEGFR2 (R2.2, *Whitaker GB, J Biol Chem 2001 Jul 6;276(27):25520-31*) at a 1:5000 dilution of each. Resulting films are scanned and quantitated using Quantity-One software (Bio-Rad) and results reported as the amount of phosphotyrosine signal over receptor signal (P-Tyr/Receptor).

In HEK293 stable cells receptor phosphorylation assays are performed in R2-6 and T2-3 cells that are plated, transfected and stimulated with ligand in 6 well dishes. Each well is lysed with 100µL of TLB and 20ug of the resulting protein is loaded onto a 6% SDS-PAGE gel, transferred to PVDF and serially western blotted with anti P-Tyr and anti-receptor antibodies as described above. No immunoprecipitation of the receptors was necessary.

Results: pCMV6-XL4/ HPTPbeta transfected HEK293H cells have increased phosphatase activity over mock-transfected controls as measured with DiFMUP (Figure 3A). Increased phosphatase activity is also seen in HUVEC's infected with the HPTPbeta adenovirus versus the green fluorescent protein (GFP) control adenovirus (Figure 3B). The activity in both cell types is inhibited with 500uM of the generic tyrosine phosphatase inhibitor BMOV (bismatolatol oxovanadium) (Figure 3).

Transient overexpression of HPTPbeta using the pCMV6-XL4/HPTPbeta expression plasmid decreased ligand-mediated phosphorylation of VEGFR2 and Tie-2 in HEK293-receptor stable cells as seen in the anti-phosphotyrosine western blots of immunoprecipitated receptors in from VEGFA or Ang1 stimulated cells (Figure 4). Similarly, adenoviral overexpression of HPTPbeta in endothelial cells decreased the ligand-induced phosphorylation of the endogenous VEGFR2 and Tie-2 receptors as seen in the anti-phosphotyrosine western blots of

immunoprecipitated receptors from VEGFA or Angl stimulated HUVEC's (Figure 5). Taken together, these findings indicate that HPTPbeta can negatively regulate the activation of both Tie-2 and VEGFR2. These results also demonstrate a method for measuring the effects of modulating HPTPbeta activity on Tie-2 and VEGFR2 activation. Importantly, the activation of other RTKs could be negatively regulated by HPTPbeta and these effects could also be measured by this method.

Example 4. Active and inactive forms of HPTPbeta modulate VEGF165 induced calcium flux in endothelial cells

Methods: Human endothelial cells (HUVEC) were seeded at 1 x 10^6 cells per 100 mm dish in EGM one day prior to infection with adenovirus. Individual plates were infected with either active or inactive HPTPbeta adenoviruses in EGM for 24 hours at a multiplicity of infection of 50. Inactive HPTPbeta has the active site catalytic cysteine (Cys1904) mutated to a serine (HPTPbeta-C/S). Uninfected cells were left in EGM during the infection period. After incubation with the virus, infected and uninfected cells were re-plated in 96-well plates at 10,000 cells per well for an additional 24 hours before assaying for calcium flux in response to recombinant VEGF165 (R&D Systems). Plated cells were loaded with 4μM of the calcium sensing dye FLUO-4 according to the manufacturers instructions (Molecular Probes). Intracellular calcium flux was measured using a FLIPR plate reader. All calcium responses to VEGF165 were normalized to the maximal signal stimulated by 10μM of the calcium ionophore ionomycin (Sigma).

Results: The graph in figure 6 shows a decrease in the magnitude of the VEGF165 stimulated calcium flux in wild type HPTPbeta infected cells (▲) compared to uninfected controls (■). Conversely an increase in VEGF stimulated calcium flux was seen in the cells infected with a catalytically inactive form of HPTPbeta(●). No change in EC_{50} for VEGF165 stimulated calcium flux was observed only changes in apparent maximal signal. These findings demonstrate that HPTPbeta can regulate signaling by VEGF receptors and that a downstream signaling assay such as calcium mobilization could be used to test the efficacy of HPTPbeta modulators in endothelial cells.

Example 5. Knockdown of HPTPbeta protein with an antisense oligonucleotide enhances ligand-induced VEGFR2 and Tie-2 receptor phosphorylation

Methods: Antisense (AS) oligonucleotides capable of selective inhibition of HPTPbeta expression are supplied by Sequitur, Inc. (Natick, MA). Design and screening of the HPTPbeta antisense

oligonucleotides (AS) is accomplished as follows: ten phosphorothioate-containing DNA oligonucleotides with perfect 25 nucleotide complementarity to selected regions of the HPTPbeta coding sequence were synthesized by standard phosphoramidite chemistry and purified with trityl-on using reverse-phase columns followed by de-salting on a size exclusion matrix. Chemistry-matched control oligomers, lacking significant sequence homology to known or predicted human genes, are made in parallel. Antisense and control oligonucleotides are transfected into Human Microvascular Endothelial Cells with Lipofectamine 2000 using conditions recommended by the manufacturer (Invitrogen, Inc.). Uptake efficiency is monitored in live cells using a fluorescently labeled oligonucleotide as described by the manufacturer (Sequitur, Inc.). At 24h post-transfection, cells are lysed and polyadenylated mRNA is isolated with an mRNA Catcher™ plate (Sequitur, Inc.). Levels of HPTPbeta mRNA are determined by Real Time RT-PCR (Taqman™) and normalized to GAPDH mRNA. The PCR primers and probe used for HPTPbeta amplification hybridize upstream of the predicted sites of AS oligo annealing. Three AS oligos elicited potent inhibition of HPTPbeta mRNA showing an average 73% decrease in HPTPbeta mRNA levels as compared to chemistry matched oligo controls (Antisense oligos available from Sequitur, Inc.- part numbers S17924, S17929, and S17930).

Transfection in HUVEC's is performed with the above-mentioned antisense oligos at a 200nM final concentration using Lipofectin (Invitrogen) in OptiMEM-I (Gibco) according to the manufacturers instructions. After a 2-hour transfection, the cells are returned to normal growth media (EGM, Clonetics) and after 48 hours, assayed for VEGFR2 and Tie-2 receptor activity as described in example 4. Uptake efficiency is monitored in every transfection using a fluorescently labeled oligonucleotide and determined to be >90% in every experiment.

Results: Western blotting of VEGF stimulated HUVEC's show significant increases in the level of VEGFR2 and Tie-2 phosphorylation when HPTPbeta protein levels are decreased by 60%. (Figure 7) Taken together, these findings further support the findings in Example 4 and demonstrate that endogenous HPTPbeta can negatively regulate the activation of both Tie-2 and VEGFR2. These findings also demonstrate that the activity of HPTPbeta inhibitors could be measured using a receptor activation assay.

Example 6. Changes in HPTPbeta expression modulate VEGF induced endothelial morphogenesis in the microbead sprouting assay

Methods: Evaluation of HPTPbeta overexpression in the micro-bead sprouting assay is performed by culturing $2x10^6$ HUVECs with 5 mg of Cytodex beads (Sigma) in 10 ml of EGM (Clonetics) in 100mm bacteriological dishes for 48 hours. Cell coated beads are transferred to a 50 ml conical tube washed and resuspended in 380 ul D-PBS. Collagen matrix is prepared by adding 71.4 ul of rinsed cell coated beads to 2.8 mL of a collagen matrix solution consisting of 8 volumes of 3 mg/ml rat tail collagen (Sigma) in 0.1% acetic acid, 1 volume of 10x M-199 supplemented with 100 mg/ml L-glutamine, and 1 volume of 10x collagen buffer (0.05N NaOH, 200 mM HEPES, 260 mM $NaHCO_3$). Three hundred and fifty microliters (350uL) of the mixture is then dispensed immediately into a well on a 24 well tissue culture plate and the matrix was allowed to solidify for 45 minutes at 37°C/5% CO2. Afterwards 1 ml of EGM with or without 50 ng/ml VEGF was added per well and returned to the incubator. After 24 hours, the media was replaced with fresh EGM with or without VEGF (50 ng/ml), correspondingly. Forty-eight hours after matrix formation, a blinded observer visualized the sprouts with a phase contrast inverted microscope and observed 50 beads per well, in quadruplicate wells, for the presence of endothelial cell sprouts. Results are expressed as the number of sprouts per bead. To determine the effect of HPTPbeta overexpression, HUVEC coated beads are infected with an HPTPbeta or GFP adenovirus at 100 MOI in EGM 18 hours before plating in matrix. To evaluate the effects of HPTPbeta knockdown, cells attached to the beads were transfected with 200nM antisense oligos using Lipofectin (Invitrogen) according to the manufacturers protocol for 2 hours in the original non-coated dish at 37°C/5% CO_2 before being introduced to the matrix. The transfected cell coated beads are transferred to a 50 ml conical tube, rinsed once with Dulbecco's phosphate buffered saline (D-PBS, Gibco) and resuspended in 3 ml D-PBS. Fibrin gels are prepared by adding 400 µl of rinsed cell coated beads to 1.6 mL of a matrix solution consisting of 2 mg/mL fibrinogen, 200 U/mL aprotinin and 2.5 U/mL thrombin in D-PBS with or without 10ng/mL $VEGF_{165}$ (R&D Systems). Four hundred microliters (400µL) of the beads are then dispensed immediately into a well on a 24 well tissue culture plate and the matrix was allowed to solidify for 10 minutes at 37° C/5% CO_2. Afterwards 500 µl of EGM was added per well and returned to the incubator. After 1 hour, the media is replaced with fresh EGM with or without VEGF (10 ng/ml), correspondingly, and again after 24 hours. After 48 hours, a blinded observer visualized the sprouts as described above.

Results: HPTPbeta overexpression decreases VEGF-induced sprouting as compared to GFP infected controls in HUVEC's (Figure 8A). Conversely, antisense knockdown of HPTPbeta enhances VEGF induced endothelial cell sprouting (Figure 8B). These findings confirm the regulation of VEGFR2

by HPTPbeta in examples 4 and 5 and demonstrate that the angiogenic activity of VEGFR2 is modulated by HPTPbeta in parallel with receptor activation. These findings also demonstrate that this assay could be used to measure the effect of HPTPbeta inhibitors on endothelial cell sprouting in the absence or presence of additional growth factor.

Example 7. Adenoviral-mediated overexpression of HPTPbeta attenuates angiogenesis in the rat aortic ring explant assay.

Methods: The isolated rat aortic ring model of angiogenesis is performed using the thoracic aorta from 150-175 gram, male Sprague-Dawley rats (Charles River). The excised thoracic aorta is placed in sterile saline solution and transferred to endothelial basal media (EBM, Clonetics) containing 1% antibiotics/antimycotic (Gibco BRL). Periadventitial fibroadipose tissue and any visible blood clots were removed. Aortas are cross-sectioned at ~1mm intervals and the resulting tissues placed in 100 µl of Dulbecco's Modified Eagle Medium (DMEM) containing 2% FBS, in the presence or absence of adenovirus for green fluorescent protein, empty virus or HPTPbeta (all at 1.0 x 10^{11} virus particles/ml), as described. Rings are incubated for 30 minutes at 37°C in a humidified incubator under a constant 5% CO_2 atmosphere. Following adenoviral infection, aortic rings were washed with EBM and placed in a 24-well tissue culture plate. Aortic rings are overlaid with 450 µl of collagen solution consisting of 8 volumes of 3 mg/ml rat tail collagen (Sigma) in 0.1% acetic acid, 1 volume of 10x M-199 supplemented with 100 mg/ml L-glutamine, and 1 volume of 10x collagen buffer (0.05N NaOH, 200 mM HEPES, 260 mM $NaHCO_3$). The collagen solution is allowed to solidify for 1 hour and covered with 1 ml of EBM. Images (TIFF) are taken using a SPOT camera connected to a Nikon inverted microscope and analyzed (vessel extent and area) using automated image analysis software.

Results: Rat thoracic aortic rings are infected with empty virus, GFP containing virus or HPTPbeta containing virus showed that the parameters of vessel extent and area were significantly reduced in HPTPbeta infected rings (Figure 9). These data demonstrate that HPTPbeta can negatively regulate blood vessel formation in a model that has been shown to be dependent on VEGFA.

Example 8. A selective HPTPbeta inhibitor (Compound 1 ((R)-[1-Methylcarbamoyl-2-(4-sulfoamino-phenyl)-ethyl]-carbamic acid tert-butyl ester) enhances the ligand-induced VEGR2 and Tie2 receptor autophosphorylation.

Methods: The potency of a class of HPTPbeta inhibitors was optimized by iterative screening for inhibition of HPTPbeta activity measured as described in Example 2. This iterative screening process resulted in the discovery of a nanomolar HPTPbeta inhibitor. To determine selectivity, compound 1 was tested against a panel of several other recombinant phosphatases (Figure 10). VEGFR2 and Tie-2 receptor activation was assayed as mentioned in example 4. However, to assess the effect of the HPTPbeta inhibitor, the cells were pretreated for 30 minutes prior to stimulation with either 0.1 mM or 1 mM Compound 1 in Opti-MEM media (Invitrogen) at 37°C/5%CO_2.

Results: A potent and selective selective HPTPbeta inhibitor (Compound 1) was developed (see Example 11) as an agent to enhance the activation of VEGFR2 and Tie2 (Figure 10). Compound 1 was at least 10 fold selective for HPTPbeta versus the any of the other phosphatases except for the closely related HPTPeta. As seen in Figure 11, anti-phosphotyrosine western blotting revealed an increase in VEGFR2 and Tie-2 receptor activation following a 30-minute pre-treatment with a small molecule inhibitor of HPTPbeta. Clearly, these assays could be used to optimize the potency and selectivity and to test the efficacy of other HPTPbeta inhibitors.

Example 9. A Selective HPTPbeta inhibitor increases ligand induced endothelial cell survival

Methods: Human umbilical vein endothelial cells (HUVEC, Clonetics) were seeded at 10,000/well in a 96-well plate for 2 hours in EGM media (Clonetics). The cells were then serum-deprived for 2 hours in DMEM (Invitrogen) supplemented with 0.2% bovine serum albumin. Quadruplicate wells were then treated with either vehicle or 500μM Compound 1 in the presence or absence of either 500pM recombinant human $VEGF_{165}$ or 250 ng/mL recombinant human Angiopoietin-1 (Ang-1) (R&D Systems) for 72 hours in DMEM supplemented with 0.2% bovine serum albumin. Cells remaining after incubation were detected using the CellTiter-Glo™ System according to the manufacturers instructions (Promega) and measured using a Victor V plate reader (Perkin Elmer/Wallac).

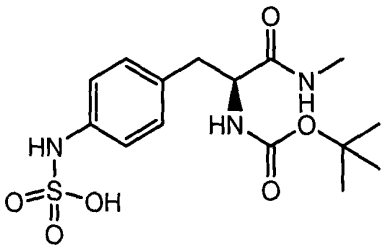
Results: As seen in Figure 12, endothelial cells treated with 500μM of the HPTPbeta inhibitor Compound 1 show increased cell survival when stimulated with 500pM $VEGF_{165}$ or 250 ng/mL Angiopoietin-1 (Ang-1). Interestingly, Compound 1 also increased basal levels of survival over untreated controls.

Example 10. A small molecule inhibitor of HPTPbeta increases in vascular sprouting in the ex-vivo rat aortic ring model.

Methods: The rat aortic ring model was essentially performed as described in example 7 with the exception that increasing concentrations of Compound 1 was added to the final 1mL of EBM added on top of the solidified collagen mixture and was present for the remainder of the experiment.

Results: Rat thoracic aortic rings treated at the time of plating with increasing concentrations of a small molecule inhibitor of HPTPbeta demonstrate significant increases in the parameters of vessel extent and area with 100μM and 300μM Compound 1 (figure 13).

Example 11. Synthesis of Compound 1 ((R)-[1-Methylcarbamoyl-2-(4-sulfoamino-phenyl)-ethyl]-carbamic acid tert-butyl ester)



Compound 1 ((R)-[1-Methylcarbamoyl-2-(4-sulfoamino-phenyl)-ethyl]-carbamic acid tert-butyl ester)

Boc-Phe(4-NO_2)-NMe: Boc-Phe(4-NO_2)-OH (10.0 g, 32.3 mmol) is dissolved in anhydrous tetrahydrofuran (32.2 mL) with 4-methylmorpholine (3.90 mL, 35.4 mmol). Isobutylchloroformate (4.18 mL, 32.3 mmol) is dropwise added at 0°C and the mixture is stirred for 1 hr. at 0°C. Methylamine (332.3 mL, 2.0 M in tetrahydrofuran) is added dropwise at 0°C and the mixture is stirred for 18 hr. at room temperature. The mixture is then recrystallized from 1:1 DCM:methanol to give 6.69 g pure white solid.

Boc-Phe(4-NH_2)-NMe: Boc-Phe(4-NO_2)-NMe (500 mg, 1.55 mmol) is dissolved in methanol (10 mL). To this was added palladium on carbon (10% by weight, 50 mg). The reaction is placed under a hydrogen atmosphere until reaction is complete (tlc). The catalyst is removed by filtration and the filtrate is concentrated to provide the amine, which is used without purification.

(R)-[1-Methylcarbamoyl-2-(4-sulfoamino-phenyl)-ethyl]-carbamic acid tert-butyl ester: In a dry flask 0.420g of the aniline compound is dissolved in 2 mL pyridine. To this solution is added 0.684g

of sulfurtrioxide-pyridine complex. The mixture is stirred 5 minutes then diluted with 25 mL of 7% ammonium hydroxide. The mixture is evaporated down to an off-white solid and purified to provide 0.106g of product as its ammonium salt. $^1H(D_2O)$: δ 7.04 (s, 4H), 4.07-4.05 (m, 1H), 2.92-2.68 (m, 2H) 2.55 (s, 3H), 1.24 (s, 9H)

Miscellaneous

Except as otherwise noted, all amounts including quantities, percentages, portions, and proportions, are understood to be modified by the word "about", and amounts are not intended to indicate significant digits.

Except as otherwise noted, the articles "a", "an", and "the" mean "one or more".

All documents cited are, in relevant part, incorporated herein by reference; the citation of any document is not to be construed as an admission that it is prior art with respect to the present invention.

While particular embodiments of the present invention have been illustrated and described, it would be obvious to those skilled in the art that various other changes and modifications can be made without departing from the spirit and scope of the invention. It is therefore intended to cover in the appended claims all such changes and modifications that are within the scope of this invention.